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Short Communication

Kallikrein-related peptidase signaling in colon carcinoma cells: targeting proteinase-activated receptors

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Abstract

We hypothesized that kallikrein-related peptidase 14 (KLK14) is produced by colonic tumors and can promote tumorigenesis by activating proteinase-activated receptors (PARs). We found that KLK14 is expressed in human colon adenocarcinoma cells but not in adjacent cancer-free tissue; KLK14 mRNA, present in colon cancer, leads to KLK14 protein expression and secretion; and KLK14 signals via PAR-2 in HT-29 cells to cause (1) receptor activation/internalization, (2) increases in intracellular calcium, (3) stimulation of ERK1/2/MAP kinase phosphorylation, and (4) cell proliferation. We suggest that KLK14, acting via PAR-2, represents an autocrine/paracrine regulator of colon tumorigenesis.

Keywords: cancer; colon; kallikrein; PARs; proliferation; signaling.

Proteinases (colloquially termed 'proteases') have fundamental roles in multiple biological processes and are associated with a wide variety of pathological conditions including cancers (Mook et al., 2004; Lopez-Otin and Matrisian, 2007). Serine proteinases have recently emerged as extremely important signaling molecules that are involved in human tumor progression by targeting diverse substrates that favor all steps of tumor evolution. Accumulating evidence indicates that serine proteinase family of the kallikrein-related peptidases (KLK) are dysregulated in cancer (mainly in adenocarcinomas), and their up-regulation is often associated with poor patient prognosis (Borgono and Diamandis, 2004; Obiezu and Diamandis, 2005; Pampalakis and Sotiropoulou, 2007; Emami and Diamandis, 2008; Sotiropoulou et al., 2009; Lawrence et al., 2010). Although primarily known for their clinical applicability as cancer biomarkers, recent evidence implicates KLKs in many cancer-related processes, including cell growth regulation, angiogenesis, invasion, and metastasis (Borgono et al., 2004; Sotiropoulou et al., 2009). It has been shown that KLKs can affect cell function by generating active peptides from precursor polypeptides, activating other proteinases, and cleaving cell adhesion molecules (Borgono and Diamandis, 2004; Veveris-Lowe et al., 2005; Borgono et al., 2007; Sotiropoulou et al., 2009). In addition, KLKs have emerged as signaling molecules by selectively targeting members of a unique subfamily of cell surface G-protein-coupled receptors termed 'proteinase-activated receptors' (PARs) (Oikonomopoulou et al., 2010b).

PARs are activated by cleavage of their extracellular amino terminus, at a specific locus, by tryptic serine proteinases that unmasks a 'tethered' ligand sequence that binds to the receptor extracellular domains to trigger signaling (Coughlin, 2005; Hollenberg et al., 2008; Adams et al., 2011). Originally, PAR-1, PAR-3, and PAR-4 were thought to be targeted only by thrombin, whereas PAR-2 is activated by trypsin and other trypsin-like serine proteinases but not by thrombin. Like the tethered ligands, short synthetic peptides (activating peptides, or APs) corresponding to the newly exposed amino-terminus are able to activate a given PAR receptor selectively and mimic cellular effects of the proteinase in the absence of proteolytic cleavage (Vu et al., 1991; Dery et al., 1998; Hollenberg and Compton, 2002; Adams et al., 2011). Although, the coagulation proteinase thrombin can be seen as a prototype physiological activator of PARs (signaling via PAR-1, PAR-3, and PAR-4), other serine proteinases, apart from the coagulation proteinases that activate PARs in physiological settings *in vivo*, have yet to be identified. In the small intestine, trypsin is likely to be the physiological activator of the epithelial PAR-2 (Kong et al., 1997). Mast cell tryptase, also thought to be a potential physiological activator of PAR-2 in the setting of inflammation (Corvera et al., 1997; Mirza et al., 1997), may only activate the receptor in a restricted environment where PAR-2 glycosylation is altered (Compton et al., 2002). More recently, the kallikrein-related peptidases

(KLKs) have emerged as potential regulators of PAR function (Oikonomopoulou et al., 2006a,b, 2010b).

Several lines of evidence point to an intimate link between PAR-2 expression and a variety of cancers including melanoma (Shi et al., 2004), breast cancer (Su et al., 2009), reproductive tissues (Osuga et al., 2008), prostate (Shi et al., 2004), and gastrointestinal tract (D'Andrea et al., 2001; Kaufmann et al., 2009) including colon cancer (Darmoul et al., 2001). PARs as well as their activators can be now considered important players in the development of human colon cancer. Indeed, we have previously demonstrated that in colonic tumors, trypsin acting, through up-regulated PAR-2, and thrombin, acting through aberrantly expressed PAR-1 and PAR-4, are very robust growth factors that can trigger MAP kinases that in turn stimulates the migration and proliferation of human colon cancer cells (Darmoul et al., 2001, 2004a,b; Gratio et al., 2009). However, the endogenous tumor- or stroma-derived proteinases that can be responsible for activating PARs in colon cancer remain unknown.

Using pharmacological approaches, it has been found that members of the kallikrein-related peptidase family (KLK5, KLK6, and KLK14) are potential PAR activators. It has thus been suggested that in tissues that express these enzymes, KLKs may regulate tissue function via triggering the PARs (Oikonomopoulou et al., 2006a; Stefansson et al., 2008). In prostate cancer cells, KLK4 and KLK2 have been found to stimulate MAP kinase signaling along with increases in intracellular calcium (Mize et al., 2008; Ramsay et al., 2008). Very recently, we demonstrated that KLK4 selectively activates PAR-1 signaling, but not signaling via the other PARs, in colon cancer cells (Gratio et al., 2010). Other work has documented the ability of KLK5, KLK6, and KLK14 to activate calcium signaling via PAR-2 (Oikonomopoulou et al., 2006a; Stefansson et al., 2008). As elevated KLK14 levels have been found to be associated with unfavorable prognosis in patients with colon cancer (Talieri et al., 2009a), we hypothesized that tissue KLK14 itself may represent an important regulator of the PARs, which are known to be up-regulated in colon cancer.

We investigated the hypothesis that KLK14 can signal via PAR-2 in colon carcinoma using various means: by immunohistochemistry, the expression of KLK14 in colonic tumors; by polymerase chain reaction, the presence of KLK14 mRNA in colon tumor-derived cell lines and normal colonic epithelial cells; by ELISA, KLK14 secretion in conditioned media of colon tumor-derived cell lines; by microscopic analysis, the effect of KLK14 on PARs internalization from cell surface of HT-29, a cell line that express both PAR-1 and PAR-2; and the analysis of ERK1/2/MAP kinases phosphorylation as a biological response to KLK14, which leads to cell proliferation in human colon cancer cells. The data we report here, as presented at a recent 4th International Symposium on Kallikreins and Kallikrein-Related Peptidases (ISK2011) (Rhodes, Greece: September 2–4, 2011) complement and support other observations we have made in a parallel study that supports a role for KLK14 in colon cancer oncogenesis via its activation of PAR-2 (Gratio et al., 2011).

As a prelude to our work with the human colon tumor-derived cell line HT-29 cells, we first analyzed the expression of KLK14 in colon cancer from patients with adenocarcinomas. As shown in Figure 1, KLK14 expression was found in the dysplastic mucosa (Figure 1B) and also in the cancerous lesions (Figure 1C,D). In these specimens, staining was localized in the cytoplasmic compartment of the apical part of the epithelial cells and in some stromal cells. The intensity of staining, however, varied from case to case (not shown). In contrast, minimal if any staining for KLK14 was observed in epithelial cells in 'normal' human colonic mucosa that is remote from the neoplastic colon cancer lesions (Figure 1A). Analysis of 40 cases did not reveal any correlation between the level of expressed KLK14 in the cancerous epithelium and the site of the tumor within the colon, type of tumor, tumor stage, or degree of tumor differentiation (data not shown).

Although originally known for its value as a biomarker in breast, ovarian, and prostate cancer (Obiezu and Diamandis, 2005), we are the first to provide evidence for the differential expression of KLK14 detected immunohistochemically in colon cancer compared with its absence in the normal human colonic epithelia. Our data are in accord with another study (Shaw and Diamandis, 2007) documenting the low or absent level of KLK14 in normal adult colon using ELISA and RT-PCR approach. However, that study found a detectable level of KLK14 in fetal colon tissue that may mimic a 'dedifferentiated' state of adult colon cancer tissue. Our cell-targeted approach, using immunohistochemistry to show a marked expression of KLK14 in the epithelial cells of adenocarcinoma tissues and its absence in the glandular epithelial cells in adjacent normal colon, is more suitable to detect significant localized cancer-associated changes of KLK14 levels, compared with studies that have been based on whole tissue extracts for analysis (Yousef et al., 2004; Ogawa et al., 2005; Feng et al., 2006; Talieri et al., 2009b). That said, using multivariable statistical analysis, elevated KLK14 levels were found to be associated with unfavorable survival prognosis in patients with colon cancer (Talieri et al., 2009a). These observations thus show, for the first time, that human colonic adenocarcinomas aberrantly express high levels of KLK14.

Following our analysis of colon cancer tissues, the expression of KLK14 transcripts was investigated in human colon cancer cell lines by RT-PCR analysis. As shown in Figure 2, KLK14 mRNA is present in all human colon cancer cells analyzed (Figure 2A) with weaker expression of KLK14 detectable in HT-29Cl.19A and Caco-2 cells. Under comparable RT-PCR conditions, KLK14 mRNA was not detectable with confidence in epithelial cells isolated from normal human colon (Figure 2B). The faint signal detected in the normal epithelial cells may have come from stromal cells that may have contaminated the epithelial cell preparation. Indeed, the presence of stromal cells is suggested by the appearance of a faint band of vimentin mRNA, whose expression is restricted to stromal cells and not found in epithelial cells (von Bassewitz et al., 1982), whereas the epithelial cell content of the colon-derived sample was evidenced by the presence of villin mRNA, a specific epithelial cell marker (Pringault et al., 1991). It is worthy of note that the RT-PCR data are in agreement with the

immunohistochemical data showing a lack of expression of KLK14 in normal vs. up-regulated expression in the colonic adenocarcinoma tissue (Figure 1).

The levels of KLK14 in various cell line supernatants were then quantified using an immunoassay of the conditioned media from human colon cancer cells grown *in vitro*. As shown in Figure 3, KLK14 protein is secreted by many human colon cancer cell lines. High KLK14 levels (110±15 ng/l) were observed in the conditioned media from SW480, T84, and SW48 cell lines. Lower levels were seen in HT-29, Caco-2, LoVo, and LS174T cell lines. KLK14 immunoreactivity in other colon cancer-derived cell lines (SW620 and HCT-116) was very low or undetectable (not shown). These data reveal that quite a number of colon cancer-derived cell lines express and secrete immunoreactive KLK14 extracellularly. Because KLK14 is secreted as a zymogen (Borgono and Diamandis, 2004; Sotiropoulou et al., 2009; Lawrence et al., 2010) it is difficult to correlate the levels of immunoreactive KLK14 with the concentrations of active enzyme in the conditioned media. Indeed, in ovarian cancer ascites fluid, although substantial levels of a KLK can be detected by immunoassay (e.g., KLK6), only a small, albeit biologically significant proportion, is enzymatically active due to the presence of serine proteinase inhibitors (Oikonomopoulou et al., 2008, 2010a). However, our data showing the presence of active KLK10 in ovarian cancer ascites samples suggest that in the restricted microenvironment of a tumor *in vivo*, accumulated, secreted KLK14 could be present at concentrations sufficient to regulate PAR function in an autocrine/paracrine loop within the colonic tumor. The use of activity-based probe analyses that can quantify active KLK14 in the tumor microenvironment of colon cancer cells would be of considerable value in this regard (Oikonomopoulou et al., 2010a). It is also possible that stromal cells as well as the tumor epithelial cells themselves may be able to produce KLK14 to affect tissue function.

As it has been reported that the biological effects of KLK family members can be due to their ability to activate PARs (Oikonomopoulou et al., 2006a; Stefansson et al., 2008), we investigated whether KLK14 can signal through PAR-1 and/or PAR-2 in the HT-29 colon cancer-derived cell line. Using immunofluorescence microscopy with antibodies directed against the N-terminal domains of either PAR-1 or PAR-2, which are expressed on HT-29 cells (Darmoul et al., 2001, 2003), we examined the loss of PAR-1 and PAR-2 immunoreactivity at the cell surface of KLK14-treated HT-29 cells. This loss of immunoreactivity reflects receptor cleavage, activation, and internalization (Trejo, 2003; Ramachandran et al., 2009). As shown in Figure 4, treatment of HT-29 cells

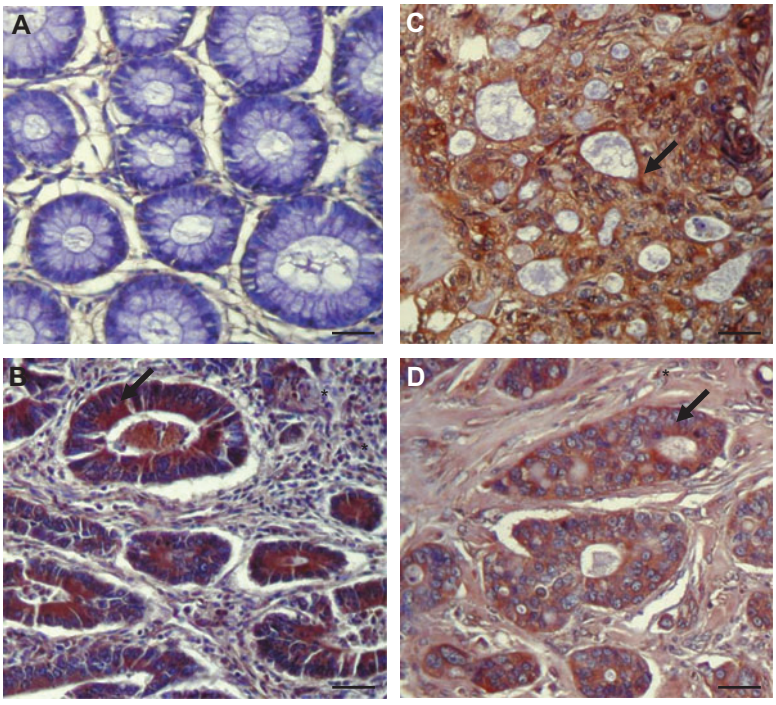

Figure 1 A representative immunostaining for KLK14 in paraffin sections of colonic tissues from patients with adenocarcinomas and adjacent normal colonic mucosa.

Dewaxed sections were overlaid overnight with the polyclonal KLK14 antibody. Specific binding was detected by the streptavidin-biotin-peroxidase method as described. Tissues were used in accordance with the requirement of the Human Research Committee of the Bichat-Claude Bernard Hospital and according to French bioethical law (French Bioethics Law, 2004). (A) Negative or discrete staining of KLK14 was found in the adjacent section of a colonic mucosa distant from an adenocarcinoma. (B–D) High and variable immunoreactivity (arrows) is seen in the epithelial cells of adenocarcinomas of three different patients. Asterisks (*) show stromal cell staining. Black arrow point to positive immunoreactivity in the cytoplasmic compartment of epithelial cells. Bar =50 μm.

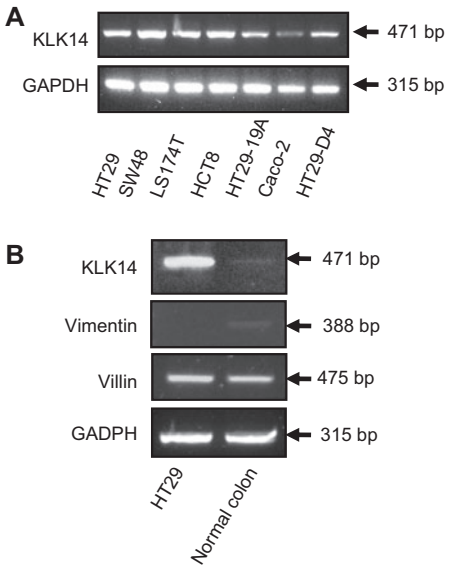

Figure 2 Expression of KLK14 mRNA in human colon cancer cell lines and normal colonic cells.
Constitutive expression of KLK14 mRNA in (A) various human colon cancer cell lines (B) HT-29 cells vs. normal human colon epithelium. Human-derived colon cancer cell lines were maintained as decribed by Darmoul et al. (2001). Colonic epithelial cells were isolated as previously described (Salomon et al., 1993). Total RNA is isolated with guanidium isothiocyanate method. Two micrograms of total RNA were reverse transcribed using oligo-dT primers. Amplifications were conducted using 25% of the cDNA mixture and human KLK14 sense primers 5′-ACA TCA AGG AGG GAC CTG TG-3′ and antisense primer 5′-GAG TTG TAG TTG GGG TGC GT-3′. Vimentin, stroma cell marker (von Bassewitz et al., 1982) was amplified using sense primers 5′-GAC AAT GCG TCT CTG GCA CGT C-3′ and the antisense primers 5′-GGG ACT CAT TGG TTC CTT TAA GGG C-3′. Villin, an epithelial cell marker (Pringault et al., 1991) was amplified using the sense primers 5′-TGT TCC TTC CAG CAC CTT TG-3′ and antisense primer 5′-CCT GAG TCT CTC CAT ACG GG-3′. GAPDH cDNA amplification was used as an internal control with sense primer 5′-TCG GAG TCA ACG GAT TTG GTC GTA-3′ and antisense primer 5′-AGC CTT CTC CAT GGT GGT GAA GA-3′. Each of the 30 cycles of amplification was performed as follows: 94°C for 40 s, 65°C for 40 s, and 72°C for 40 s. PCR products were identified by electrophoresis in 2% agarose gel followed by SyberSafe staining.

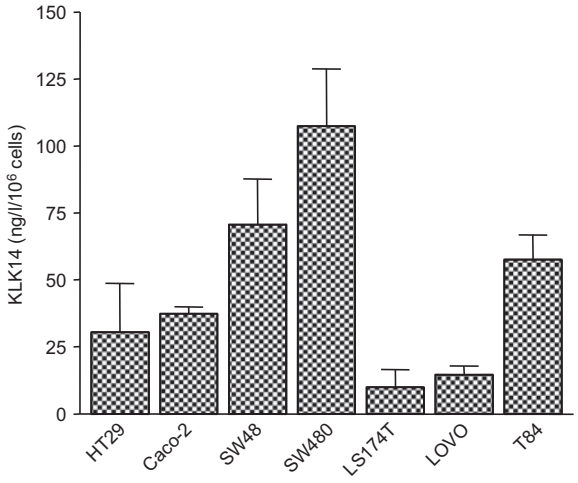

Figure 3 KLK14 secretion in colon cancer cell lines.
HT-29 cells were seeded at 500 000 cells/flask. At confluence, cells were counted and the conditioned medium was collected for measurement of KLKs. ELISA for KLK14 was performed using a noncompetitive immunoassay, as previously described (Shaw and Diamandis, 2007). Protein values represent the mean concentration of KLK14 expressed by 10^6 cells.

with KLK14 (50 U/ml) for 10 min has little or no effect on PAR-1 cell surface staining (Figure 4A). In contrast, KLK14 treatment of cells resulted in a substantial decrease of PAR-2 immunoreactivity at the cell surface (Figure 4B). The loss of PAR-2 cell surface immunoreactivity seen in KLK14-treated HT-29 cells can be due either to receptor internalization, which requires proteolytic cleavage at a specific activation site (Dery et al., 1998), or to cleavage of PAR-2 downstream of the activation site, which results in 'receptor disarming' (Oikonomopoulou et al., 2006b). Thus, to monitor receptor activation, we evaluated PAR-2 intracellular localization in permeabilized HT-29 cells following KLK14 challenge. As shown in Figure 4C, pretreatment with KLK14 induced a rapid (10 min) internalization of PAR-2, resulting in a diffuse intracellular localization of the receptor in the cytosol of KLK14-treated HT-29 cells compared with control cells. These data indicate that, in HT-29 cells, KLK14 can cleave and activate PAR-2, triggering its internalization.

As we previously reported, receptor-selective peptide agonists for PAR-1 and PAR-2 both induced increases in intracellular Ca^{2+} in HT-29 cells, verifying that HT-29 cells express functional PAR-1 and PAR-2 (Darmoul et al., 2001, 2003; Gratio et al., 2010). We therefore determined if KLK14 could trigger calcium signaling in HT-29 cells. As shown in Figure 5A, HT-29 cell challenge with KLK14 induced intracellular Ca^{2+} mobilization in a concentration-dependent manner, indicating that KLK14 can signal to HT-29 cells by increasing intracellular calcium concentrations. We used a cross-desensitization approach with PAR-1/PAR-2-selective agonists (Kawabata et al., 1999; Hollenberg and Compton, 2002) to establish the specificity of KLK14-mediated response via PAR-1 and/or PAR-2. As shown in Figure 5B, a challenge of the cells with PAR-2-desensitizing concentrations of 2-furoyl-LIGRLO-NH_2 (50 μM), a potent PAR-2-specific AP (McGuire et al., 2004; Kanke et al., 2005) essentially abolished the subsequent KLK14-induced increase in intracellular calcium, as compared with the control KLK14-induced signal (right tracing). In contrast, PAR-1 desensitization with the PAR-1-selective AP, TFLLR-NH_2, did not affect the KLK14-induced Ca^{2+} response (Figure 5C). Similarly, the KLK14-induced Ca^{2+} mobilization was not affected by a prior challenge of the cells with thrombin (not shown). These results demonstrate pharmacologically that KLK14 preferentially cleaves and activates PAR-2 (relative to PAR-1) in HT-29 cells.

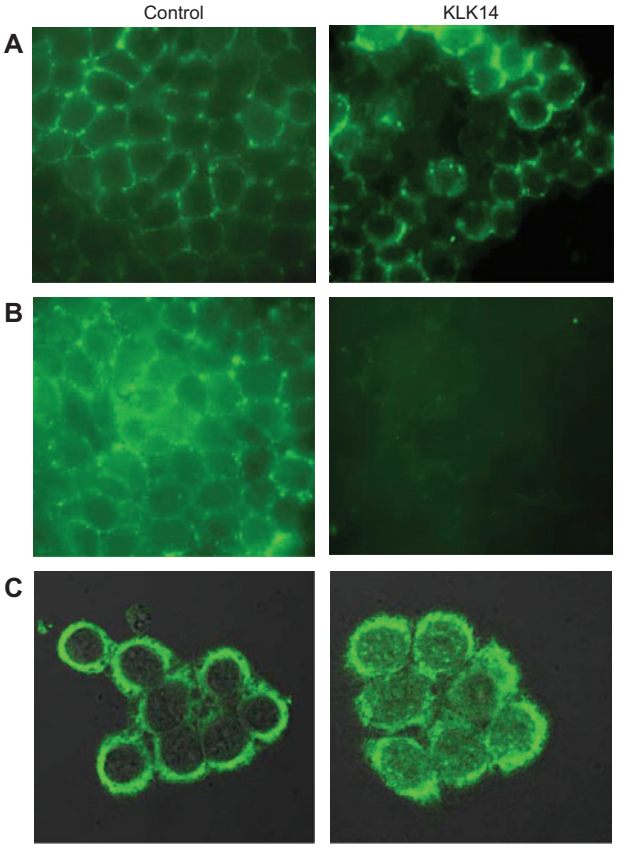

Figure 4 KLK14 induces loss of PAR-2 from the surface of HT-29 cells.
Immunofluorescence detection was performed on HT-29 cells grown on glass cover slips as described (Gratio et al., 2010) using anti-PAR-2 (mAb 13-8) and anti-PAR-1 (WEDE15) following incubation of HT-29 cells for 10 min at 37°C with either vehicle or KLK14 (50 U/ml). Cells were fixed in 2% paraformaldehyde or with cold acetone for 30 s for PAR-2 internalization studies before application of the primary anti-PAR-2 (mAb 13-8) monoclonal antibody. An Alexa Fluor 488 dye-conjugated goat antimouse antibody was used. The cells were then mounted in Vectashield medium. Images were examined under a fluorescence microscope (Leica DM IRB; magnification ×630). (A) Immunofluorescence detection of PAR-1 in HT-29 cells treated with vehicle or KLK14 (50 U/ml). (B) Immunofluorescence detection of PAR-2 in HT-29 cells treated with vehicle or KLK14 (50 U/ml). (C) Cells were fixed and permeabilized with acetone and immunostained with PAR-2 monoclonal antibody (magnification ×630). Results are representative of two independent experiments.

It has been established that trypsin, acting via PAR-2, can trigger the activation of extracellular-regulated kinase (ERK1/2/MAP kinase) in colon cancer cells and thereby stimulate cell proliferation (Darmoul et al., 2001, 2004a). Therefore, we next investigated the effect of KLK14 on ERK1/2/MAP kinase phosphorylation activation. We used concentrations of KLK14 that were catalytically equivalent (units of enzyme activity per milliliter) to the concentration of trypsin we previously used for stimulating ERK/MAP kinase phosphorylation and cell proliferation (approx.

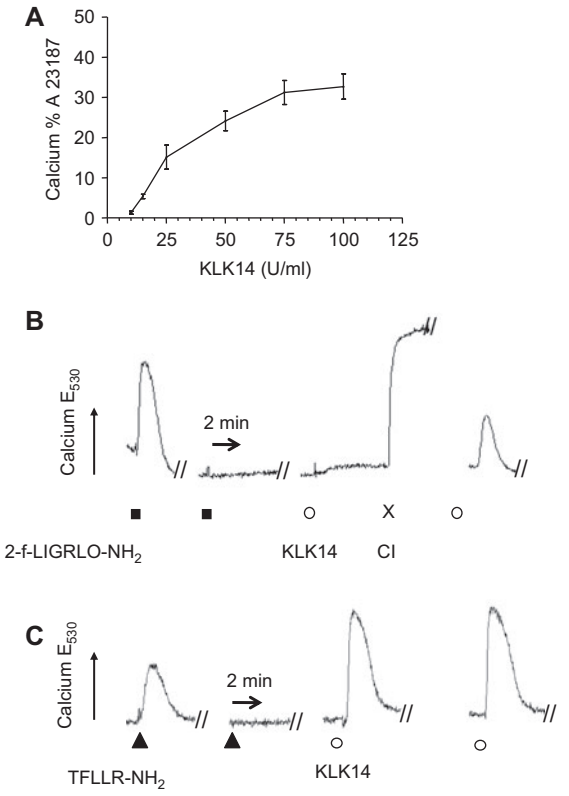

Figure 5 Mobilization of intracellular calcium by KLK14 in HT-29 cells coexpressing functional PAR-1 and PAR-2.
HT-29 cells seeded and grown to 95% confluency in 75 cm^2 T-flasks using 10 ml of Dulbecco minimal essential medium/10% v/v fetal calf serum were harvested (trypsin-free EDTA-containing isotonic saline, pH 7.4), resuspended in 1 ml of Fluo-4AM-(4 μM)-No wash (NW) calcium indicator solution (Invitrogen Canada, Burlington, ON, Canada) and incubated for 25 min at room temperature. The fluorescence signal (E530) caused by the addition of test agonists (kallikrein, or PAR-APs) was quantified as a percentage, relative to the signal generated by A23187 (2 μM) (% A23187) essentially as previously described (Kawabata et al., 1999). (A) Concentration-effect curves for mobilizing intracellular calcium in HT-29 cells by KLK14. Cells were challenged by addition of KLK14 at the indicated concentrations. The calcium signal, relative to that caused by 2 μM ionophore (A23187), was monitored for increasing enzyme concentrations. Each data point represents the average±SE (bars) for three measurements at each enzyme concentration. (B) Mobilization of intracellular calcium by KLK14 and desensitization of KLK14 action by preactivation of PAR-2 in HT-29 cells. Cells were challenged or not by two sequential additions of 2-furoyl-LIGRLO-NH_2, the PAR-2-specific AP (50 μM, ■) to desensitize PAR-2. Desensitized cells were then exposed to KLK14 (100 U/ml, ○) followed by the addition of calcium ionophore (CI, ×). The right-hand tracing shows the response of a separate aliquot of the same cells to KLK14 (100 U/ml, ○) without prior exposure to 2-furoyl-LIGRLO-NH_2. Data are representative of three independent experiments. (C) PAR-1 desensitization effect on KLK14-induced calcium response in HT-29 cells. Cells were first desensitized with two sequential additions of TFLLR-NH_2, the PAR-1-specific AP (25 μM) (▲), prior to KLK14 (100 U/ml) (○). The right-hand tracing shows the response of a separate aliquot of the same cells to KLK14 (100 U/ml, ○) without prior exposure to TFLLR-NH_2. Data are representative of three independent experiments.

0.5–1 U/ml, measured by a microtiter plate enzyme assay). As shown in Figure 6A, addition of KLK14 (1 U/ml) to quiescent HT-29 cells for 5 min induced significant phosphorylation of ERK1/2/MAP kinase comparable to that induced by known PAR-2 agonists [10 nM of trypsin or 100 μM of the PAR-2-AP (AP2) SLGKV-NH_2]. These results indicate that KLK14 activates the MAP kinases pathway in colon cancer cells similar to trypsin-mediated signaling (Darmoul et al., 2004a). The involvement of PAR-2 in KLK14 signaling was demonstrated further by the use of monoclonal antibodies directed against the sequence spanning the proteinase cleavage/activation site of PAR-2 (mAb 13-8) (Gratio et al., 2010) and using PAR-2 mRNA knockdown with siRNA. As shown in Figure 6A, blocking cleavage of PAR-2 with the monoclonal antibody antagonist mAb 13-8 inhibited the KLK14-induced ERK1/2/MAP kinase phosphorylation. As expected, the PAR-2 monoclonal antibody also inhibited the trypsin-induced ERK1/2/MAP kinase phosphorylation in the HT-29 cells, whereas it had no effect on AP2-induced ERK1/2/MAP kinase phosphorylation. These results clearly shows that KLK14 can act at the cleavage site of PAR-2 to induce cell signaling in colon cancer cells. Similarly, using siRNAs that target PAR-2 specifically, we found that the KLK14-induced ERK1/2/MAP kinase phosphorylation is strongly inhibited by PAR-2 mRNA knockdown (Figure 6B). Similarly, PAR-2 siRNAs also inhibited trypsin-induced ERK1/2/MAP kinase phosphorylation. As expected, PAR-2 siRNA had no effect on thrombin-induced ERK/MAP kinase phosphorylation mediated by PAR-1 (Darmoul et al., 2004a). These results fully support the pharmacological desensitization data demonstrating that KLK14 signals preferentially via PAR-2 in the HT-29 colon cancer cells.

Finally, we evaluated the effect of KLK14 on HT-29 cell growth. Of note, treatment of HT-29 cells with KLK14 (0.5 U/ml) significantly increased the number of HT-29 cells at 96 h poststimulation (Figure 6C). This response was comparable to that induced by either 1 nM trypsin or 100 μM of AP2 (Darmoul et al., 2001, 2004a).

Taken together, the findings summarized in this report complement and amplify data from our parallel study (Gratio et al., 2011), supporting the hypothesis that colon tumor-derived KLK14 can act in an autocrine/paracrine manner via PAR-2 to have an impact on colon cancer cell signaling and growth. Thus, not only can KLK14 be seen as a potential 'tumor biomarker' of poor prognosis in colon cancer, but the proteinase can also be seen potentially to function during the oncogenic process. What cannot be determined from our current work is whether all of the KLK14 produced in the tumor tissue remains catalytically active or is rapidly inhibited by tumor-produced (or stroma) serine proteinase inhibitors. Future work with serine proteinase activity-based probes coupled with mass spectroscopic analysis should be able to answer this question, as we have done for KLK6 and KLK10 in the setting of ovarian cancer (Oikonomopoulou et al., 2008, 2010a). Notwithstanding, our data point to two novel therapeutic targets that may be considered for the treatment of colon carcinoma: the proteinase KLK14 and (2) the G-protein coupled receptor, PAR-2. Selective antagonists for

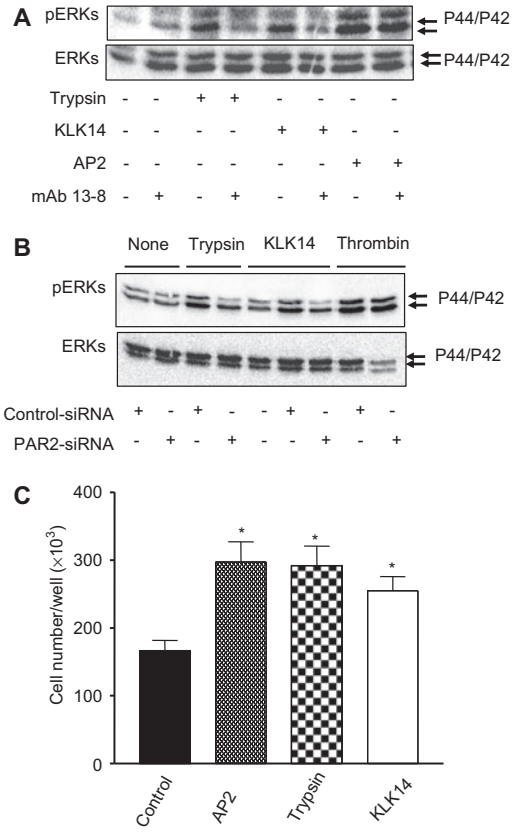

Figure 6 KLK14-stimulated p42/p44 MAPK in HT-29 cells is mediated by PAR-2.

(A) Effect of siRNA-mediated *PAR-2* gene silencing on KLK14-mediated ERK1/2 phosphorylation. HT-29 cells grown in serum-free medium were challenged for 5 min with trypsin (10 nM), SLIGKV-NH_2 (100 μM), or with KLK14 (1 U/ml) with or without a prior pretreatment of the cells with blocking antibody anti-PAR-2 (mAb 13-8) (200 nM) for 2 h. Cell lysates were then directly analyzed for ERK1/2 phosphorylation with anti-phospho-ERK1/2. The blot was subsequently stripped and reprobed with anti-ERK1/2 to verify equal protein loading lanes. The figure shows a representative immunoblot from two other experiments. (B) Effect of siRNA-mediated *PAR-2* gene silencing on KLK14-mediated ERK1/2 phosphorylation. HT-29 cells were seeded for 24 h before siRNA transfection. HT-29 cells were left untreated (control), transfected with nonsilencing control siRNA or with Smart pool siRNAs duplexes (100 nM) specific for human *PAR-2*. HT-29 cells were harvested 48 h later for determination of PAR-2 gene expression using RT-qPCR. For ERKs signaling, HT-29 cells were analyzed at day 5 after transfection and then were shifted to serum-free medium for 24 h before stimulation with agonists. Cells were challenged for 5 min with trypsin (10 nM), KLK14 (1 U/ml), or thrombin (10 nM) as a negative control. Cell lysates were then directly analyzed for ERK1/2 phosphorylation with anti-phospho-ERK1/2 antibody. The blot was subsequently stripped and reprobed with anti-ERK1/2 to verify equal protein loading among lanes. The figure shows a representative immunoblot from two experiments. (C) KLK14-induced cell proliferation in HT-29 cells. Cells were seeded in medium containing 10% FCS. After 3 days, cells were washed and covered with serum free medium for 48 h. Quiescent cells were grown for 96 h in serum-free medium without (control), with SLIGKV-NH_2 (100 μM), trypsin (1 nM), or KLK14 (0.5 U/ml). After 96 h, cells from triplicate wells were counted for each condition. Data are means±SEM of three different experiments. *$p<0.02$, KLK14, AP2-, or trypsin-treated cells vs. control cells.

both of these targets are becoming available and are worthy candidates for future evaluation as adjuncts to colon cancer chemotherapy.

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